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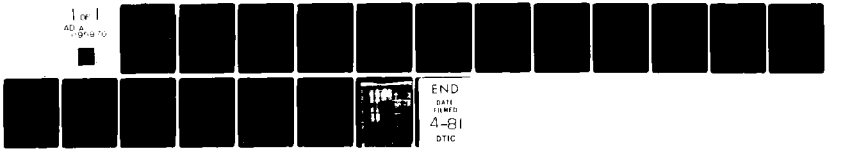

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Affinity Chromatographic Purification of Endogenous Pyrogen with Cibacron Blue[1,2]

WALTER J. CRITZ,[3] CHARLES F. MATSON, BERTHONY LADOUCEUR, AND EDWARD C. HAUER

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Fort Detrick, Frederick, Maryland 21701

Running Title: DYE-LIGAND CHROMATOGRAPHY OF EP

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Subject Category: CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES

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INTRODUCTION

Fever has a long history of association with illness, but the fact that an infected host produces a molecule (endogenous pyrogen, EP[4]) responsible for the fever was first demonstrated only in 1953 by Bennett and Beeson (1). It is now known that EP is a protein that is produced by phagocytic host cells upon stimulation, and travels via the bloodstream to the hypothalamic region of the brain, where it acts by altering the thermoregulatory "set-point."

The structural features and the mechanisms responsible for its biological activity are currently unclear. While the existence of an essential protein component of EP is well-established, it is unlikely that lipid (2) and carbohydrate (3) components are important for the observed biological activity. Rabbit, but not human, EP is inactivated by sulfhydryl reagents (4), implying the need for reduced thiol groups on at least the rabbit protein. Attempts to purify endogenous pyrogen meet difficulties due to the fact that it is present in small quantities amid a host of more abundant proteins.

Affinity chromatography with the blue dye ligand Cibacron Blue F3GA was chosen in an attempt to separate EP from proteases which might be present, and from albumin, which is a major contaminant. This paper describes the findings that in addition to proteases and albumin, EP also binds to the affinity columns, and that this binding can be used as an effective purification step.

MATERIALS AND METHODS

Cibacron Blue agarose, cm-Cibacron Blue agarose (AffiGel Blue and cm-AffiGel Blue, respectively) and BioGel P-60 were purchased from BioRad Laboratories, Richmond, California. Activated CH-Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey. Ethanolamine and Tris were obtained from Sigma Chemical Co., St. Louis, Missouri, human serum albumin, from Armour Pharmaceutical Co., Chicago, Illinois. Goat anti-human albumin and rabbit anti-bovine serum were from Miles Laboratories, Elkhart, Indiana.

Rabbit EP was obtained from rabbit peritoneal exudate cells (5). The final concentration was 10^8 cell equivalents/ml. Human EP was obtained from the hystiocyte cell line U-937, as described by Bodel et al. (6).

Rats used for the bioassay were male Fisher-Dunning 344 strain, approximately 225 g body weight, obtained from Harlan Industries, Indianapolis, Indiana. The bioassay of EP was performed by intracerebroventricular (icv) injection of the pyrogen into rats with in-dwelling catheters (7-9). A fever unit is defined as the amount of pyrogen that causes a 1°C rectal temperature increase. Plasma zinc concentrations were measured by atomic absorption as described by Bailey et al. (7).

All glassware was sterilized by autoclaving prior to use, and care was taken to prevent endotoxin contamination. Plasticware was sterile and pyrogen-free. Endotoxin was assayed by the Limulus amebocyte lysate (LAL) test, using a kit from M.A. BioProducts, Walkersville, Maryland.

Isoelectric focusing (IEF) was performed using thin-layer pH 3-10 polyacrylamide focusing gels from LKB Instruments, Rockville, Maryland.

Affinity columns were prepared with 100 g each of either Cibacron Blue agarose or cm-Cibacron Blue agarose. The gel was washed in 0.01 M potassium phosphate buffer containing 0.15 M NaCl at pH 7.2 (phosphate-buffered saline, PBS), then packed into a 2.5 x 30 cm column. This column was then equilibrated with PBS, and the samples applied. The fractions (10 ml each) were eluted at a rate of 60 ml/h. A stepwise or linear gradient (0.15-1.4 M NaCl in pH 7.2) was introduced when approximately 200 ml of PBS had been eluted, by which time the absorbance at 280 nm dropped below 0.005 absorbance units. Two-ml aliquots were withdrawn, dialyzed in PBS to bring the salt concentration to physiological ranges, and injected ic. into rats for bioassay (7-9).

Gel filtration was performed on a 2.5 x 40 cm column of BioGel P60, operated at 60 ml/h. The amount of EP used for each chromatogram was 1×10^9 cell equivalents, for the single column procedures and 1×10^{10} for two-column procedures. The elution buffer was PBS. Bovine serum albumin (67,000 daltons) myoglobin (17,000 daltons), and cytochrome c (12,000 daltons), from Sigma Chemical Co., St. Louis, Missouri, at 1 mg/ml each were used to calibrate the column. Ten-ml fractions were collected.

For preparation of the anti-albumin Sepharose, anti-bovine serum, and the albumin-Sepharose columns, 1 g of activated CH Sepharose 4B was washed and reswelled with 200 ml of 1 mM HCl. Then with the 0.1 M, pH 8.0 sodium bicarbonate coupling buffer it was packed into a 1 x 5 cm column. The albumin or antibody (5 mg), in coupling buffer was washed into the column and allowed to react overnight at 4°C. Excess albumin and unreacted gel sites were removed by washing at room temperature with 1 M pH 8.0 ethanolamine, then Tris buffer (0.05 M, pH 8.0). The coupled gel was then alternately washed three times each with 200 ml of 0.1 M acetic acid and Tris buffer, both containing 0.5 M NaCl.

The EP fractions were concentrated by ultrafiltration using YM-10 membranes from Amicon Corp., Lexington, Massachusetts.

RESULTS

The pyrogenic samples studied were determined to be EP, because they came from known EP sources, had an assay curve typical of EP (8, 9), contained the correct weight, 13,000-15,000 dalton, molecules based on gel filtration, and were heat labile. In addition, the pyrogens tested negative for endotoxin (less than 0.5 ng/ml) with the LAL assay, and were not lost during dialysis, as prostaglandins or other low molecular weight bioactive species would have been.

Binding of EP to Cibacron Blue Columns

Rabbit endogenous pyrogen was found to bind to a column of cm-Cibacron Blue, equilibrated with PBS, and to elute at high salt concentration. Fig. 1 shows the elution pattern obtained by chromatographing 2×10^8 cell equivalents of crude rabbit EP. This pattern has been found consistently to be reproducible with rabbit pyrogen. As indicated in Table 1, Peak II in the elution profile contained the pyrogen, since it induced fever in assay rats. Zinc-depressing activity, which is commonly associated with EP (5, 7), also eluted in this fraction. The data show that all the pyrogen activity applied could be accounted for in Peak II, and that approximately 5-fold purification of the crude starting material occurred in this one step.

A pattern similar to that of Fig. 1 was obtained when EP was chromatographed over Cibacron Blue rather than cm-Cibacron Blue. However, Peaks I and II were more nearly equal in area with Cibacron Blue, because more extraneous material eluted in Peak II, resulting in lower specific activity.

Some of the applied crude material bound too tightly to be removed by the 1.4 M NaCl, and was instead eluted with 6 M urea. The tightly bound material did not contain pyrogenic activity, nor did the small peak at 240 ml (Fig. 1).

cm-Cibacron Blue Gradient Chromatography of EP

Attempts were made to further improve cm-Cibacron Blue chromatography by the use of a NaCl gradient. In addition to the rabbit EP, attempts were made to use this method to improve the purity of human EP. Fig. 2 shows the effect of a linear 0.05-1.4 M NaCl gradient upon the elution of crude human and rabbit EP. In both studies, the pyrogenic activity was eluted in a broad band beginning half-way through the gradient. This activity appeared in the trailing half of the first major peak observed after the gradient started. As expected from the results of Fig. 1 and Table 1, no pyrogenic activity appeared in the peak eluted before the gradient was initiated.

Two-Step Purification of Rabbit EP

Gel Filtration through BioGel P-60 was coupled with the cm-Cibacron Blue chromatography to see how much the final purification would be improved, and to allow purification of larger amounts of EP, since it was found that amounts greater than 2×10^9 cell equivalents of crude rabbit EP overloaded the cm-Cibacron Blue Column. Table 2 shows the results of a typical experiment of this type. These two simple chromatographic procedures resulted in removal of 99% of the contaminating protein with retention of half the activity. The majority of the activity loss occurred with the gel filtration, as observed by others (10, 11).

The specific activity of the EP at the end of the these steps, measured by the rat icv assay, was 5.8×10^3 fever units/mg, which corresponded to 172 ng/fever unit. Because the rat assay is 50-100 times

more sensitive than the conventional rabbit EP assay (8, 9), this would correspond to 8-17 μg/fever unit for the latter method.

Samples at different stages of this purification scheme were isoelectric focused to assess further their relative purity. The effectiveness of the cm-Cibacron Blue column in fractionating the gel filtrate into two distinct groups of proteins is shown by comparing the IEF patterns for Peak I (Fig. 3, samples 3 and 4) and Peak II (Fig. 3, samples 5 and 6). Two major stained bands were seen in the focusing pattern of Peak II, along with several minor ones. The two major bands visible in the Peak II IEF pattern corresponded to pH values of 5 and 7.

Interaction of EP with Albumin-Sepharose

To remove albumin, human EP (0.1 mg in 1 ml, 2×10^6 cell equivalents), partially purified by the gel filtration and cm-Cibacron Blue steps outlined above, was allowed to immunoabsorb for 16 h on successive days on columns of Sepharose-linked rabbit anti-bovine serum and then Sepharose-linked goat anti-albumin. The EP activity was recovered in the void volume when the columns were eluted with PBS. This immunoabsorbed EP was then washed into a column of human albumin cross-linked to CH-sepharose 4B equilibrated with PBS. After overnight equilibration, the column was washed with the PBS equilibration buffer and the eluate assayed for EP activity. Again, all the pyrogen activity eluted in the void volume.

DISCUSSION

The results show that cm-Cibacron Blue affinity chromatography results in substantial purification of rabbit or human EP without loss of activity. EP purification schemes (3, 10) frequently include steps which result in high losses of activity. The gel filtration results shown, which are similar to data from other laboratories (3, 10, 11), are a good illustration of the problem: 10-fold purification occurs, but half of the pyrogen activity is lost. Whether the benefits of such steps outweigh the gains depends on other factors. In the use of gel filtration, either rabbit or human EP is separated from contaminating substances that are higher or lower in molecular weight. Endotoxin and prostaglandins, respectively, are substances which could interfere with the assay but would be removed by gel filtration if they were present.

The partial purification of rabbit EP achieved by the combination of gel filtration and cm-Cibacron Blue affinity chromatography is illustrated by the isoelectric focusing gels. There are two major bands at pH 5 and 7, and a number of fainter ones. It is interesting to note that the pI values of these major bands respectively match those of the trimeric and monomeric forms (3) of rabbit EP. Murphy et al. (10) reported a specific activity of 30,000 rabbit pyrogen doses/mg of rabbit EP. The rabbit EP partially purified by gel filtration and Cibacron Blue chromatography has a specific activity which corresponds (9) to about 116 rabbit pyrogen doses/mg. Thus, the EP used on the isoelectric focusing gels is still highly contaminated with other proteins, which suggests that none of the bands can be said with confidence to be only EP.

For two reasons, the possibility arose that EP binds not to the Cibacron Blue columns themselves, but to albumin adhering to the column

material. These reasons are: first, that albumin does bind Cibacron Blue columns and elute (12) in the stepwise salt gradient described above to elute EP, and second, that Mitchell et al. (13) have suggested that large administered doses of heterotypic albumin may cause fever. An attempt was therefore made to establish whether EP bound directly to the Cibacron Blue column or instead to an albumin-Cibacron Blue complex. This was done by preparing an albumin-Sepharose column and seeing if EP bound to it. The EP which had been cleared of albumin by immunoabsorption, passed through this column and appeared only in the void volume. This suggests that EP bound to the Cibacron Blue column directly, rather than as a complex with albumin.

It has previously been held (14) that binding of a molecule to Cibacron Blue columns was good evidence for the presence of a "dinucleotide fold" on the binding molecule. It now appears that this is not necessarily true, and that such binding must be interpreted more empirically (12). It is interesting to speculate that EP might bind nucleotides or dinucleotides in vivo, especially in view of the role of cyclic AMP in fever (15), but there is no corroborative evidence for such binding.

The use of Cibacron Blue derivatives for affinity chromatography of rabbit and human EP is a useful technique that can increase purity 5- to 10-fold in one step without loss of pyrogen activity.

FOOTNOTES

[1]In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2]The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

[3]To whom correspondence should be addressed.

[4]Abbreviations used: EP, endogenous pyrogen; icv, intracerebroventricular; LAL, Limulus amebocyte lysate; IEF, isoelectric focusing; PBS, phosphate buffered saline.

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TABLE I

EFFECT OF CRUDE AND cm-CIBACRON BLUE-FRACTIONATED RABBIT EP IN FISHER-DUNNING RATS

Sample (n = 4)	Serum Zn^{++} concentration (mg/dl ± SE)	Temperature increase (°C/dose ± SE)	Specific activity (°C/mg ± SE)	Total protein (mg)
Saline	95.8 ± 10.9	0.24 ± 0.22	0	0
Crude rabbit EP	57.5 ± 7.1*	1.48 ± 0.20*	118 ± 16	3.56
Peak I	87.6 ± 6.5	0.55 ± 0.36	ND	ND
Peak II	60.2 ± 9.4*	1.73 ± 0.18*	590 ± 61	0.71

*$P \leq 0.05$ by student's t-test vs. saline control.

TABLE 2

PURIFICATION TABLE FOR RABBIT EP

Sample	Concentration (mg/ml)	Volume (ml)	Total Protein[a] (mg)	Total activity[b] (Fever units x 10^{-4})	Yield (%)	Purification (-fold)
Crude EP	1.7 ± 0.05	100	170 ± 5	2.0 ± 0.1	100	1
Gel filtrate	0.33 ± 0.05	50	16.7 ± 2.3	1.0 ± 0.1	50	10.2
cm-Cibacron Blue Peak II	0.7 ± 0.05	2.8	1.96 ± 0.1	1.14 ± 0.1	55	86.7

[a] Absorbance at 280 µm.

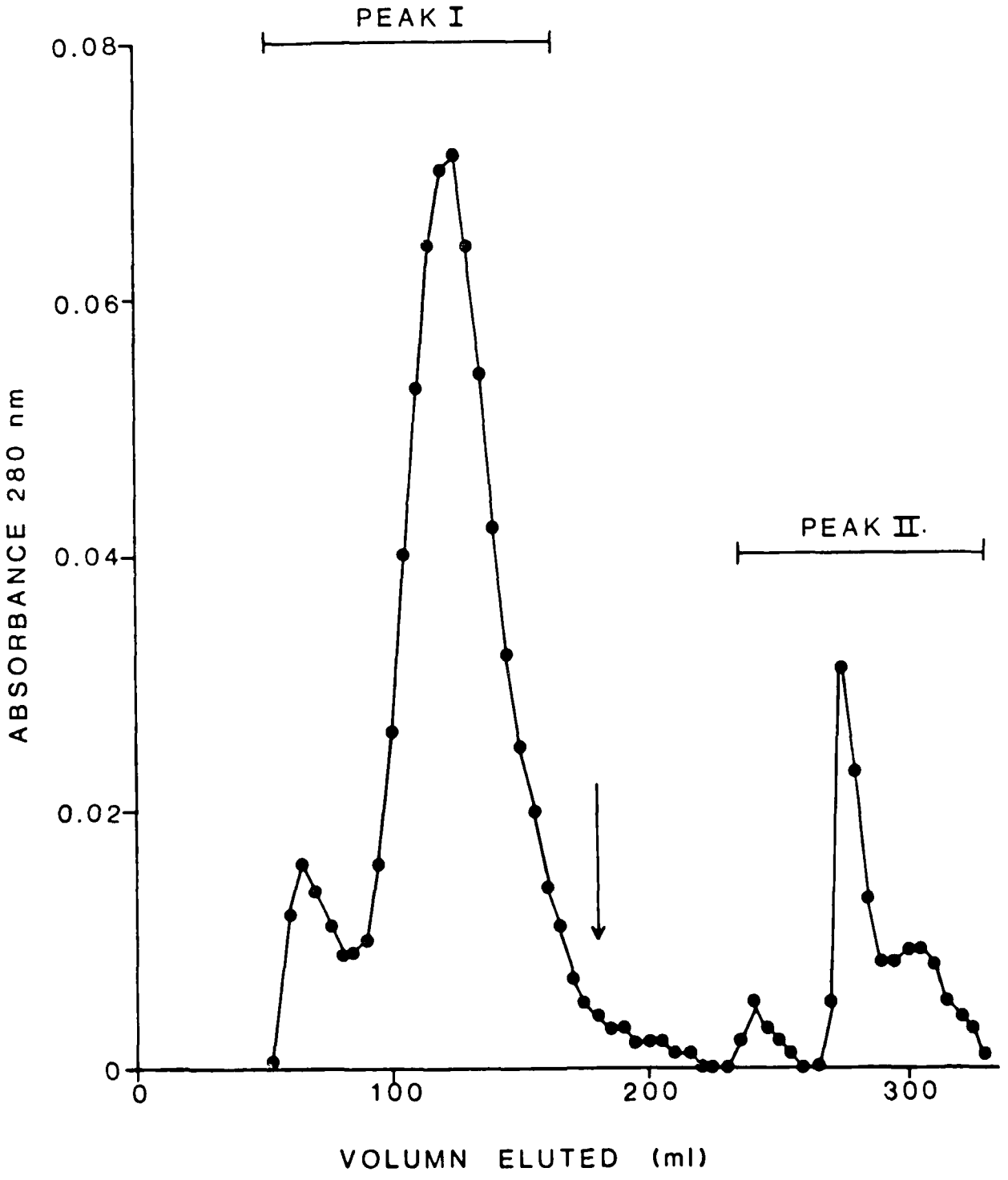
[b] A fever unit is the amount of pyrogen that causes a 1°C temperature increase in assay rats.

FIGURE TO LEGENDS

FIG. 1. cm-Cibacron Blue chromatography of rabbit EP. Crude rabbit EP (2 ml, 2 x 10^8 cell equivalents) was applied to the affinity column and washed successively with 200 ml phosphate buffered saline and then (arrow) 1.4 M NaCl. Absorbances at 280 nm vs. buffer are shown for the 10-ml fractions collected.

FIG. 2. Salt-gradient elution of rabbit and human EP from cm-Cibacron Blue agarose. Absorbance at 280 nm (solid line), rectal temperature increases (bar graphs), and the 0.05-1.4 M NaCl gradient (dotted line) are displayed for the 10-ml fractions collected. For the fever assay, 0.05 ml was injected icv. The temperature increases are those of the assay rats (n = 4) above those of the control rats (n = 4) receiving 0.05 ml of heat-inactivated EP.

FIG. 3. Isoelectric focusing patterns of rabbit EP. A thin-layer polyacrylamide gel containing pH 3-10 ampholines was focused with the following samples: 1, Crude Rabbit EP; 2, Gel filtrate; 3, Peak I, cm-Cibacron Blue column; 4, Peak I, cm-Cibacron Blue column, 1:5 dilution; 5, Peak II, cm-Cibacron Blue column; and 6, Peak II, cm-Cibacron Blue column, 1:5 dilution, the anode (pH 3) is at the top of the gel and the cathode (pH 10) at the bottom.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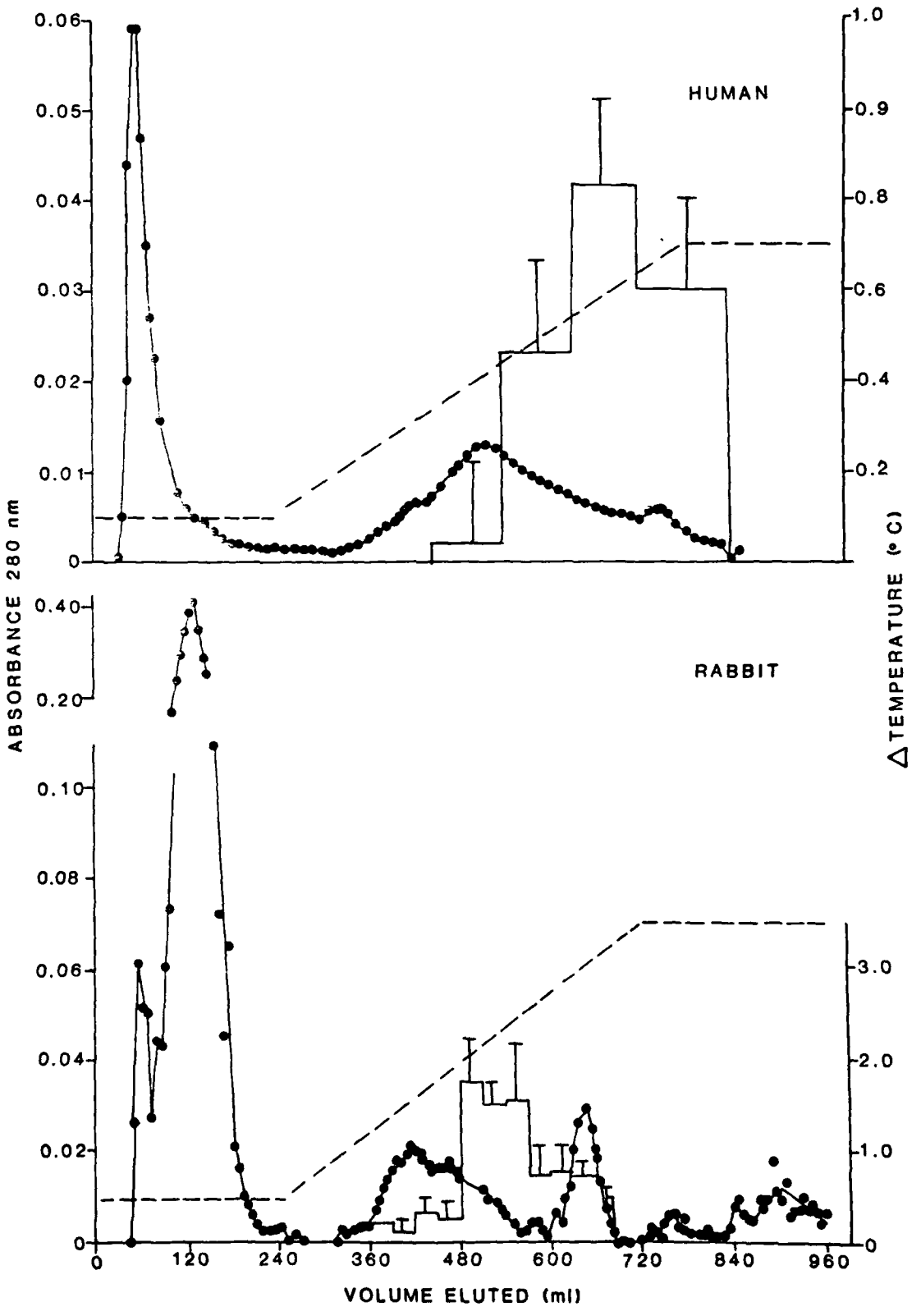
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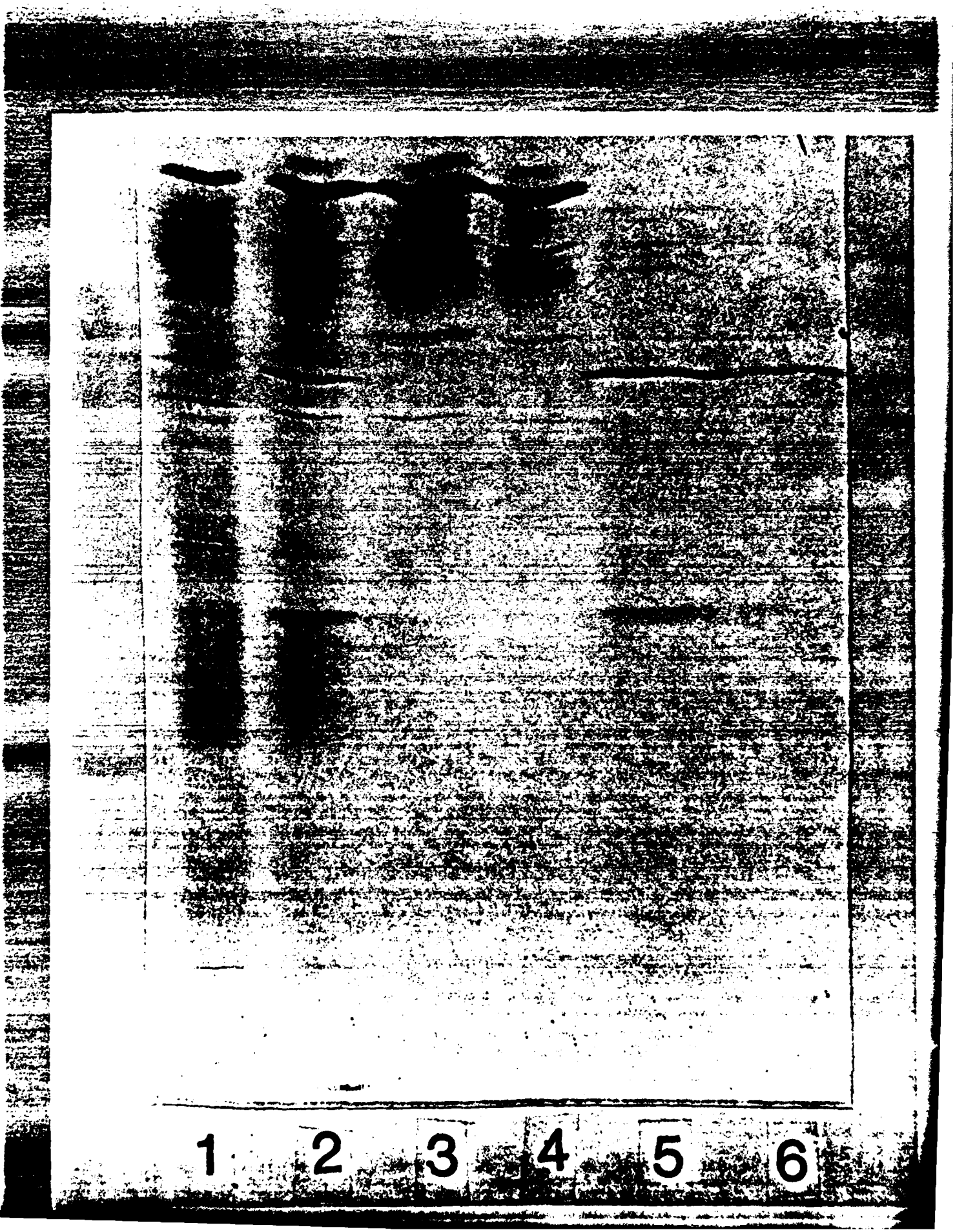
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